COMPOSITIONS AND METHODS FOR GENERATING SHORT DOUBLE-STRANDED RNA USING MUTATED RNASE III

BACKGROUND OF THE INVENTION

5

RNA interference (RNAi) employing short double-stranded RNA (siRNA) is a powerful tool for silencing gene expression (WO 01/29058, WO 01/68836, WO 01/75164). Large fragments of double-stranded RNA (dsRNA) elicit a non-specific response in mammalian cells through activation of the interferon (IFN) response pathway that leads to suppression of translation and cell death (Yang, et al., *Mol. Cell. Biol.* 21:7807-7816 (2001) and Wianny, et al., *Nat. Cell Biol.* 2:70-25, 25-33 (2000)). The standard method for generating siRNA is based on chemical synthesis of a pre-determined short sequence. In addition to the high cost of this method, computer models are generally required to predict the short sequences effective for RNAi experiments.

20

A mixture of short lengths of dsRNA obtained through partial digestion of large dsRNA with RNase III in the presence of magnesium ion buffer has been shown to "knock-down" the expression of cognate genes in cultured mammalian cell lines via RNAi (Yang, et al., *Proc. Nat'l. Acad. Sci. USA* 99:9942-9947 (2002)). However, achieving partial digestions yielding the right size range of product is often a difficult and time-consuming process and requires gel separation to obtain fragments of the desired size. Furthermore the inclusion of all possible sequences contained in the starting material is not ensured. US published

application US-2004-0038278, herein incorporated by reference, describes how RNase III in the presence of transition metal ions can produce a heterogeneous mixture of fragments of a size suitable for gene silencing. This is a significant improvement on existing methods of making siRNA fragments. However, it would be desirable to increase the flexibility of the methodology for example to permit non-critical incubation times that can vary in a broad range as determined by the convenience of the experimenter and/or to increase the range of buffers that might be used to create a heterogeneous siRNA mixture.

SUMMARY

In an embodiment of the invention, a method is provided for preparing a heterogeneous siRNA (hsiRNA) mixture that includes the step of reacting a preparation of dsRNA with an effective amount of a mutant RNase III to produce the hsiRNA mixture. This reaction may occur in a magnesium or manganese buffer.

In any of the embodiments described herein, the mutant RNase III may be characterized, for example, by a mutation in the position corresponding to E38 in *E.coli* RNase III, such as E38A, E38T, E38W or by a mutation at E65, for example, E65A in *E. coli* RNase III.

In an embodiment of the invention, a method of forming an hsiRNA preparation is described which includes the step of

-3-

combining a large dsRNA with a mutant RNase III for an
effective time period so as to cleave the large dsRNA to form the
hsiRNA preparation wherein (i) at least 90% of the large dsRNA
is cleaved as determined by gel electrophoresis and ethidium
5   bromide staining; and (ii) at least 30% of the cleaved dsRNA
which forms the hsiRNA preparation has a fragment size of 18-
30 nt. The effective time period may be from about 1 min to 20
hours, for example, 10 minutes or more, 5 hours or more or 10
hours.

10

In any of the methods described herein, the large dsRNA
has a length of at least about 50 nt.

In an embodiment of the invention, a method is provided
15   of down-regulating gene expression of a target gene, having the
following steps: (a) preparing a heterogeneous siRNA mixture
containing dsRNA fragments from a preparation of large dsRNA
by means of a mutant RNase III; (b) causing selected dsRNA
fragments from the siRNA mixture to degrade mRNA transcribed
20   from the target gene; and (c) down-regulating gene expression
of the target gene. At least one of step (a) and (b) may occur *in
vivo*. In those circumstances, the *in vivo* step occurs in a
eukaryotic cell wherein the eukaryotic cell may be present in a
mammal or, more generally, in a non-human animal such that
25   reducing expression of the one or more target genes cause a
phenotypic change. The phenotypic change may provide a
treatment for a disease in the mammal or an enhancement of a
desired characteristic in the mammal or may be diagnostic for a
selected phenotype. Down-regulating gene expression can be

-4-

used as a tool for analyzing a biochemical pathway in which the gene product functions. The biochemical pathway may be further analyzed in combination with a diagnostic reagent, for example, a diagnostic reagent that is one or more antibodies.

5

In an embodiment of the invention, step (a) of the method described above may further include combining a first hsiRNA mixture with one or more additional hsiRNA mixtures for down-regulating gene expression.

10

In an embodiment of the invention, a method is provided for selecting individual siRNA fragments from hsiRNA mixtures and introducing the individual siRNA fragments into a eukaryotic cell for down-regulating gene expression.

15

In an embodiment of the invention, an hsiRNA preparation is provided in which at least 30% of the preparation comprises fragments of a size of 18-30 nt, the preparation containing more than 10 different sequence fragments, the preparation being

20   capable of down regulating targeted gene expression in a cell wherein the targeted gene is selected from the group consisting of Akt1, 2, 3 Erk1, 2, Msk 1, p38, IRS1, PKR, PTEN, CREB, ERa, ERb, DAX, p53, DNMT1, DnMT3B, DnMT3A, TRIP, Rb, MeCP2, Caspase3, La, Furin, EGFP, RFP, Ffluc and Renilla.

25

In an embodiment of the invention, a composition is provided that includes a mutant RNaseIII containing one or more mutations wherein one mutation is located at a position corresponding to E38 in *E. coli* RNase III in which the glutamic

acid (E) has been mutated to an alanine (A). The composition may further include a large dsRNA.

BRIEF DESCRIPTION OF THE FIGURES

5

Figure 1 shows a cartoon of an alignment of examples of the 3 classes of RNAse III enzymes - human dicer (Hs Dicer); Drosophila Drosha, (Dm Drosha); S. cerevisiae RNAse III (Sc RNT1); *E. coli* RNAse III (Ec RNAse III). Top line - amino acid

10   residue numbers. Class designations as I, II and III with corresponding dsRNA product size in base pairs. Protein domains designated as follows: helicase, stippled; PAZ, brick; RNA binding domain (RBD), checkered; RNA cleavage domain (RCD), black; RNA cleavage domain with one or more

15   substitutions at homologous residues, grey. "Product" refers to the size of the dsRNA fragments produced by the unmodified enzymes specified in a buffer containing magnesium ions.

Figure 2 shows an amino acid alignment of various

20   bacterial and yeast RNAse III enzymes (SEQ ID NOS:9-25) Residues highlighted:
        in black -100% conserved among all examples shown;
        in gray - conserved among a majority of examples shown;
        star - residues proposed to be in contact with dsRNA

25   (*Structure* 9:1225 (2001)).

Figure 3 shows the activity of E38A mutant RNAse III (E38A).

A His-tagged E38A RNAse III fusion was purified by standard Nickel resin affinity purification and protein concentration determined by standard methods. Digestion of 0.5 mg of substrate MalE dsRNA (900bp) with a serial dilution of

5   E38A mutant RNase III in 50mM NaCl, 10mM TRIS HCL, 10mM MgCl2, 1mM DTT pH 7.9 (NEB Buffer 2) (New England Biolabs, Inc., Beverly, MA) at 37°C, for 1 hr is shown. The reaction mixtures were then electrophoresed through 20 % polyacrylamide gel and visualized via staining with Ethidium

10   Bromide.

Figure 3A: 0.5 µg dsRNA substrate was cleaved with the following amounts of E38A mutant RNAse III: 0.03, 0.06, 0.125, 0.25, 0.5 , 1, 2, and 4 µg (lanes 2-9) for one hour at 37°C in a

15   volume of 20µl where 10µl was loaded in each well.

Lane 1 - no enzyme.

Lane 10 - synthetic dsRNA 22-mer (size marker).

Lane 7 incubation of 0.5µg of dsRNA with 1µg of enzyme resulted in complete digestion. (Complete digestion is here

20   defined as no dsRNA substantially larger than the 22bp band observed by gel electrophoresis) .

Lane 9 shows that the amount of 22 bp product is ~60% of the dsRNA substrate. dsRNA was quantitated by densitometry using known amounts of synthetic dsRNA 22-mer to construct

25   the standard curve.

Figure 3B: Digestions were set up as in Figure 3A but were incubated overnight, demonstrating enhanced stability of the

approximately 22 bp product over an extended period of time in the presence of the complete RNAse III containing mixture.

Lane numbers correspond to enzyme conditions in Figure
3A.

Figure 4 shows the results of digestion with E38A mutant RNase III under the conditions described for Figure 3.

Figure 4A: Digestion of 0.5 μg of dsRNA with 4 μg of mutant RNase III in a volume of 20μl of buffer containing 10 mM of Zinc, Nickel, Manganese, Cobalt or Magnesium salts.

Figure 4B: Digestion of 0.5 μg of dsRNA with series of two fold dilutions of the 4 μg mutant RNase III in a volume of 20μl of buffer containing 10 mM Mn salt:

Lane 1 - no enzyme.

Lane 10 - synthetic dsRNA 22-mer.

Figure 5 shows a time course reaction using 4 μg of 900bp MalE dsRNA and 32 μg of E38A RNAse III in NEB Buffer 2, 160 ml total volume, at 37°C. 10 ml samples were removed at times indicated - 1 hour, 1 day, 2 days, 3 days, 4 days and 5 days and reactions stopped by the addition of EDTA to a final concentration of 25mM.

1st lane on left of gel contains a synthetic 22 bp dsRNA size standard;

Last lane on right of gel contains the product of a 5 day mock-digested dsRNA.

Figure 6 shows the results of an enzyme reaction performed as described for Figure 5 for times indicated in minutes.

5   From left to right: synthetic 22-mer dsRNA size marker; 1, 5, 10, 20, 30, 40 and 50 minute digestions and undigested dsRNA.

Figure 7 shows the results of an enzyme reaction

10   performed as described for Figure 3.

Lane 1, mock digestion of synthetic dsRNA 50-mer;
Lane 2, E38A digestion of synthetic dsRNA 50-mer;
Lane 3, synthetic dsRNA 22-mer.

15

Figure 8 shows a comparison of different commercial enzyme kits for cleaving dsRNA compared with E38A mutant RNase III. Substrate for each was 0.5μg of 900 bp MalE dsRNA; conditions for commercial kits are according to manufacturer's

20   instructions. Reaction conditions for E38A are as described for Figure 3. '-' mock reaction; '23' - synthetic dsRNA 23-mer. Percentiles are product yields for those reaction conditions in lanes indicated. The product yields of dsRNA fragments were quantitated as described for Figure 3.

25

Figure 8A: Silencer™ siRNA Cocktail Kit (RNAse III) (Ambion, Austin, TX).

Figure 8B: Dicer - recombinant dicer (human) (Stratagene, La Jolla, CA).

Figure 8C: ShortCut (RNaseIII)- ShortCut RNAi Kit (New England Biolabs, Inc., Beverly, MA).

Figure 8D: E38A - Left panel shows serial dilution titer starting at 4 μgs (right) and ending in 0.5 μgs (left). Shorter digestions were done using reaction conditions shown in designated lane (boxed) for the following times in minutes - 1, 5, 10, 20, 30, 40, 50, 60.

The comparison shows the digestion products of Dicer and E38A mutant RNase III differ in that a significant portion of large dsRNA has not been cleaved in the Dicer digest whereas for E38A, there is no observable large dsRNA. This difference means that unlike for the Dicer cleavage product, no further separation step is required for the E38A mutant cleavage product for removing large dsRNA prior to transfection of cells.

Figure 9 shows RNA interference activity of Firefly luciferase dsRNA cleaved with either ShortCut RNase III or E38A. Firefly luciferase dsRNA was prepared and digested with either ShortCut RNAse III according to manufacturer's instructions, or with E38A according to conditions described for Figure 3.

NIH 3T3 cells were plated on day one in 24 well plates at 50% density and allowed to grow overnight. Cells were

transfected on day 2 with 0.3 mgs per well of a pGL3 vector containing the firefly luciferase gene as a reporter in addition to one containing renilla luciferase as a control using Fugene as the transfection reagent according to manufacturer's instructions.

5   On day 3, luciferase dsRNA prepared by either ShortCut RNAi kit (black bar) or E38A (gray bars) or buffer (white bar) was transfected in the cells. Cells were harvested on day 4 and assayed for luciferase activity using Promega's Dual Luciferase Assay Kit according to manufacturer's instructions (Promega

10   Corporation, Madison, WI).

       Figure 10 shows dsRNA cleaved by the E65A mutant RNaseIII. His-Tagged E65A mutation of RNAse III was purified and quantitated as described for Figure 3.

15

       Figure 10A: Digestion of 0.5 mgs of MalE dsRNA according to conditions described in Figure 3 with a serial dilution of E65A mutant RNAse III in NEB Buffer 2 starting a 4 mgs in lane 8 and ending at 32 ngs in lane 1 (New England Biolabs, Inc., Beverly,

20   MA).
       Lane 9 - synthetic dsRNA 22-mer.

       Figure 10B: Reactions conditions of Lanes 4 and 8 in Figure 10A were repeated and digestions allowed to go for 16

25   hours.

       Figure 11 shows activity of E38T and E38W mutants. His-Tagged E38T and E38W mutations of RNAse III were purified and quantitated as described for Figure 3.

Digestion of 0.5 μg of MalE dsRNA (900bp) with serial
dilutions of E38T (Figure 11A) and E38W (Figure 11B) mutations
of RNAse III in NEB Buffer 2 at two fold dilutions of 4 μg of
mutant enzyme starting in lane 10 (Figure 11A) and lane 9
5   (Figure 11B) (New England Biolabs, Inc., Beverly, MA).
Digestions were conducted at 37°C for 30 minutes. Mock
digestions are shown in lane 2 (Figures 11A and 11B) and
overnight digestions using 4 μg of each mutant shown in lane 1
(Figure 11A and 11B). dsRNA size standards are in lane 11
10   (Figure 11A) and lane 10 (Figure 11B).

Figure 12 shows an alignment of RNase III from *Aquifex
aeolicus* and *E. coli*, highlighting regions and specific amino
acids of the protein thought to interact with dsRNA and be
15   involved with cutting of the dsRNA (Blaszczyk, J., et al.
Structure 9:1225 (2001)). Numbers indicate residue number of
the amino acid to its right. Top row shows sequence of RNase III
from *A. aeolicus*; second row shows sequence of wt RNase III
from *E. coli*. All rows below show sequences of *E. coli* RNase III
20   mutants which were assayed for activity. Mutant designations
are listed in Column 1. Activity of each mutant listed in Column
2 - wt - wild type activity, 23 - activity produces dsRNA
fragments of about 23 nt. i - inactive mutant residues are boxed
and highlighted; gray, inactive; black, producing 23-mer dsRNA;
25   no highlight, wild type. *E. coli* residues: 38, 45, 65, 117
correspond to *A. aeolicus* residues 37, 44, 64, 110, respectively.

Figure 13 shows a cartoon depiction of activity of RNAse
III E38A and E117D. Top panel - E38A (gray hexagons) binds

dsRNA (parallel lines) and cleaves it yielding a dsRNA 23-mer. Middle panel - E117D (black hexagons) binds dsRNA and does not cleave. Bottom panel - proposed result from mixing E38A and E117D in one reaction. The dsRNA would be cleaved yielding a dsRNA ladder composed of multimers of the 23-mer product of E38A alone. The size ladder can be shifted up or down by varying the ratios of the two mutants.

Figure 14 shows an E38A/E117D mixing experiment. E38A and E117D were mixed in varying ratios, placed on ice then used in dsRNA digestions under conditions described in Figure 3, 4 mgs of total enzyme was mixed with 0.5 µg of dsRNA.
Lane 1, 2 - dsRNA size standards, sizes shown;
Lanes 3 - 13, digestions using ratios shown on top of gel;
Lane 14 - mock digestion.

Figure 15 shows a cartoon depicting activity of RNAse III WT and E117D. Top panel - WT (white hexagons) binds dsRNA (parallel lines) and cleaves it yielding a dsRNA 11-mer or smaller. Middle panel - E117D (black hexagons) binds dsRNA and does not cleave. Bottom panel - proposed result from mixing WT and E117D in one reaction. The dsRNA would be cleaved yielding two sets of products: 11-mers or smaller products that would not be seen on a gel, and a dsRNA ladder composed of multimers of the 23-mer product resulting from E38A mutant RNaseIII digestion alone. The size ladder can be shifted up or down by varying the ratios of the two mutants.

Figure 16 shows a WT RNaseIII mixed with the mutant
E117D in a mixing experiment. WT and E117D were mixed in
varying ratios, placed on ice then used in dsRNA digestions
under conditions described in Figure 3. 4 mgs of total enzyme
was mixed with 0.5 mg of dsRNA. Lane 1, 2 - dsRNA size
standards, sizes shown; Lanes 3 - 13, digestions using ratios
shown on top of gel; Lane 14 - mock digestion.

Figure 17 shows output for human P53 sequence analyzed
according to the algorithm in Example VI.

DESCRIPTION OF THE EMBODIMENTS

We report the selective generation of dsRNA fragments
suitable for effective silencing of gene expression by means of
digestions with RNase III mutants in the presence of standard
buffers containing magnesium ions (Figures 3-11) or manganese
ions. Different types of mutants are described including: single
point mutations altering RNA binding or cleavage residues and
double point mutants. Examples of mutants are provided in the
figures (Figure 12) and examples (see, for example, Example 1)
that have comparable or improved activity to that described for
wild type RNase III in a manganese-containing buffer (US
published application US-2004-0038278).

The mutant RNase IIIs can be produced recombinantly
with a high yield. These enzymes are convenient to use for
reasons that include: the time of incubation of enzyme with
substrate is not critical and the enzyme does not need to be

titrated to determine a specific concentration for each use. These features make the mutant RNase III especially useful for high through-put reactions. The dsRNA product obtained after mutant RNaseIII digestion has been shown here to be effective

5   in gene silencing.

It has been demonstrated here that mixing a wild type or active mutant RNase III with an inactive mutant (E117D) can result in the formation of nucleic acid size ladders as

10   demonstrated in Figures 13 to 16. In Figure 13, E38A mutant RNaseIII and E117D are reacted with template DNA to produce products of varying size. This is because E117D binds to template but does not cleave the substrate so as to block potential cleavage sites for E38A. This effect is demonstrated in

15   Figure 14. Similarly, wild type RNaseIII and E117D are mixed together, a ladder of different sized fragments are observed (Figure 16).

The following terms as used in the description and in any

20   accompanying claims have been defined below. These definitions should be applied unless the context in which the terms are used requires otherwise.

"hsiRNA mixture" refers to a heterogeneous (h) mixture

25   of short double-stranded RNA fragments containing at least one fragment (siRNA) suitable for silencing gene expression. The RNA fragments in the hsiRNA mixture consistently contain a substantial fraction (greater than about 15% of the total number of fragments) having a length of 18-25 base pairs as determined

by ethidium-stained native polyacrylamide gel analysis. The presence of fragments larger than 25 nucleotides or smaller than 18nt is not excluded. The hsiRNA mixture is preferably obtained by digesting "large" dsRNA with a mutant RNAseIII.

"Silencing" refers to partial or complete loss-of-function through targeted inhibition of gene expression in a cell and may also be referred to as "knock down". Depending on the circumstances and the biological problem to be addressed, it may be preferable to partially reduce gene expression. Alternatively, it might be desirable to reduce gene expression as much as possible. The extent of silencing may be determined by any method known in the art, some of which are summarized in International Publication No. WO 99/32619 incorporated herein by reference. Depending on the assay, quantitation of gene expression permits detection of various amounts of inhibition, for example, greater than 10%, 33%, 50%, 90%, 95% or 99%.

"Large double-stranded RNA" refers to any dsRNA or hairpin having a double-stranded region greater than about 40 base pairs (bp) for example, larger than 100 bp or more particularly larger than 300 bp. The sequence of a large dsRNA may represent one or more segments of one or more mRNAs or the entire mRNAs. The maximum size of the large dsRNA is not limited herein. The dsRNA may include modified bases where the modification may be to the phosphate sugar backbone or to the nucleotide. Such modifications may include a nitrogen or sulfur heteroatom or any other modification known in the art. The dsRNA may be made enzymatically, by recombinant techniques

-16-

and/or by chemical synthesis or using commercial kits such as MEGASCRIPT® (Ambion, Austin, TX) and methods known in the art. An embodiment of the invention utilizes HiScribe™ (New England Biolabs, Inc., Beverly, MA) for making large dsRNA. Other methods for making and storing large dsRNA are described in International Publication No. WO 99/32619.

The double-stranded structure may be formed by self-complementary RNA strand such as occurs for a hairpin or a micro RNA or by annealing of two distinct complementary RNA strands.

"Heterogeneous" in the context of an hsiRNA mixture refers to dsRNA fragments having non-identical sequences produced from a single large dsRNA or a mixture of large dsRNAs after cleavage with RNase III or mutants thereof. The fragments collectively contain sequences from the entire length of the large RNA and hence form a heterogeneous mixture.

"RNase III" refers to a naturally occurring enzyme or its recombinant form. The RNase III family of dsRNA-specific endonucleases is characterized by the presence of a highly conserved 9 amino acid stretch in their catalytic center known as the RNase III signature motif (see below). Mutants and derivatives are included in the definition. The utility of bacterial RNase III described herein to achieve silencing in mammalian cells further supports the use of RNases from eukaryotes, prokaryotes viruses or archea in the present embodiments based on the presence of common characteristic consensus

sequences. Embodiments of the invention do not preclude the use of more than one RNase to prepare an RNA fragment mixture. Any RNase can be used herein where the RNase contains the amino acid consensus sequence

5   [DEQ][kRQT][LM]E[FYW][LV]GD[SARH] (PROSITE: PDOC00448 documentation for the RNase III). While not wishing to be bound by theory, it is here suggested that there is a region in an RNAse III of this type that specifically contacts substrate RNA. This region includes 4 specific amino acids and it is here shown

10   that a mutation in at least one particular amino acid of this region results in increased activity of the RNase III for purposes of producing dsRNA fragments. Figure 1 shows characteristic functionalities of RNases, Figure 2 shows conserved sequences in RNases from different sources and Figure 12 shows a variety

15   of mutations in different regions of the RNase III tested by applicants.

      The designation for the mutants are assigned by an amino acid position in a particular RNase III isolate. These amino acid

20   positions may vary between RNase III enzymes from different sources. For example, E38 in *E. coli* corresponds to E37 in *Aquifex aeolicus*. The position E38 in *E.coli* and E37 in *A. aeolicus* correspond to the first amino acid position of the consensus sequence described above and determined by aligning

25   RNase III amino acid sequences from the public databases by their consensus sequences. Embodiments of the invention are not intended to be limited to the actual number designation. Preferred embodiments refer to relative position of the amino acid in the RNase III consensus sequence.

Mutations in the RNAse III refer to any of point mutations, additions, deletions (though preferably not in the cleavage domain), rearrangements (preferably in the domain linking regions). Mutations may be at a single site or at multiple sites in the RNase III protein. Mutations can be generated by standard techniques including random mutagenesis and targeted genetics. Example 1 gives one approach to making mutants but this approach is not intended to be limiting.

Examples of mutants include E38A, E38T, E38W and E65A which produced a 23 bp product. E65A, E38T, E38W as determined by gel electrophoresis differed from E38A with respect to stability in a 16 hour incubation (see Figure 11) but were at least as effective as wild type RNase III in generating dsRNA fragments. E38A, E38T, E38W and E65A mutants produced an increased yield of the 23 bp product as compared to wild type RNase III in Mg^{2+} buffer. While not wishing to be bound by theory, it is postulated here that E38A binds and cleaves dsRNA template and is not then readily disassociated from the dsRNA product. The complex appears to be relatively stable and the dsRNA product is not available for further cleavage by RNAse III, wt or mutant. This may explain why mutant RNase III digestion can be performed overnight without causing the entire sample to be cleaved to small fragments such as 11nt fragments.

"Complete digestion" refers to an RNaseIII reaction in which fragments of dsRNA of a size greater than about 30 base

pairs (excluding digested material retained in the loading well or bound to enzyme) are at such low concentration that are they are not readily detected on an ethidium bromide stained 20% polyacrylamide gel.

"Host cell" refers to cultured eukaryotic cells or cells in animals, including vertebrates such as mammals including humans, and invertebrates such as insects. Host cell also refers to cells from plants and microorganisms.

"Overlapping" refers to when two RNA fragments have sequences which overlap by a plurality of nucleotides on one strand, for example, where the plurality of nucleotides (nt) numbers as few as 2-5 nucleotides or by 5-10 nucleotides or more.

"Complementary sequence" refers to a sequence which is not necessarily 100% identical to a sequence to which it hybridizes but nevertheless is capable of hybridizing to a specified nucleic acid under stringent conditions where stringent conditions may include: 400mM NaCl, 40mM PIPES pH 6.4, 1mM EDTA, 50°C or 70°C for 12-16 hours followed by washing. Sequence variations can be tolerated such as those that arise due to genetic mutation, strain polymorphism, evolutionary divergence or chemical modifications.

"Part or all" of a messenger RNA refers to that part of the messenger RNA (mRNA) which is complementary to a large dsRNA.

"Substantial portion" refers to the amount of sequence of a large dsRNA represented in sequences contained in an hsiRNA mixture. In one embodiment, the representative sequence is greater than 20%. In other embodiments, the representative sequence may be greater than 30%, 40%, 50%, 60%, 70%, 80% or 90%.

"One or more dsRNAs" refers to dsRNAs that differ from each other on the basis of sequence.

"Target gene or mRNA" refers to any gene or mRNA of interest. Indeed any of the genes previously identified by genetics or by sequencing may represent a target. Target genes or mRNA may include developmental genes and regulatory genes as well as metabolic or structural genes or genes encoding enzymes. The target gene may be expressed in those cells in which a phenotype is being investigated or in an organism in a manner that directly or indirectly impacts a phenotypic characteristic. The target gene may be endogenous or exogenous. Such cells include any cell in the body of an adult or embryonic animal or plant including gamete or any isolated cell such as occurs in an immortal cell line or primary cell culture.

The term "stable preparation" is used here and in the claims to describe a preparation of template dsRNA which is cleaved in the presence of a mutant RNaseIII where at least 30% of the starting material is cleaved into fragments having a size of about 18-30nt more particularly about 21-23 nt and the

cleavage product is stable for more than 2 hours, more particularly stable for 5 hours more particularly stable for 16 hours. Stability relates to the absence of detectable change in the size profile and amount of RNAse III reaction product on a gel at a specified time. Stability may be a relative measure observed by comparing mutant to wild type RNAse III reaaction products

The introduction of an hsiRNA mixture into vertebrate, invertebrate, plant or protoplast cells, or micro-organisms may be achieved directly into the cell or introduced extracellularly into a cavity or interstitial space, into the circulation of an organism, orally, by bathing, transdermally, by a transmucosal route, topically or by use of viral vectors to infect the host with the DNA.

Standard protocols of transfection or transformation may be used for introducing dsRNA into cells in culture, for example, protocols using transfection reagents in the NEB catalog (New England Biolabs, Inc., Beverly, MA) or Lipofectamine 2000, Oligofectamine (Invitrogen, Carlsbad, CA), TRANS-IT TKO® (Mirus Corp., Madison, WI), Targefect (Targeting Systems, Santee, CA), calcium phosphate or electroporation. Engineered vectors containing fragments from hsiRNA or siRNA can include bacterial vectors, plasmids or viral vectors for transforming or transfecting whole organisms. A gene gun may be utilized for plants for directing dsRNA into chloroplasts for example. The methodology for introducing foreign nucleic acids into organisms and cells is well known in the art. Introduction of the dsRNA

mixture via DNA clones expressing individual fragments from a particular dsRNA mixture into whole animals can be achieved by means of standard techniques for introducing nucleic acids.

In this specification and the appended claims, "a", "an" and "the" is not intended to be limited to a single form unless the context clearly dictates otherwise.

Advantages of the methods described herein include:

(a) obtaining rapidly (within minutes) an enhanced concentration of dsRNA fragments of a size suitable for silencing of gene expression by a rapid, cost effective process that is not dependent on a further size separation step. The methodology provides dsRNA fragment preparations from large dsRNA that contain a plurality of overlapping dsRNA fragments in which less than about 20% are uncut large dsRNA and more about 30% have a fragment size of 18-30 base pairs more particularly 21-23 bp. In preferred embodiments of the method, as much as 60% of the large dsRNA is cleaved into fragments of 21-23 nt. Because of its simplicity, this approach is amenable to automation and high throughput;

(b) forming a preparation of dsRNA fragments with gene silencing activity without requiring identification of the particular fragment giving rise to the gene silencing effect where the preparation is substantially stable without further purification for an extended period of time relative to existing enzymatic approaches;

(c)   providing a means to utilize the products of the method by cloning individual fragments or forming libraries or arrays of clones to enable mapping these fragments with respect to the RNA from which they are derived as well as testing individual fragments for gene silencing activity where the libraries may span the entire genome if required; and for *in vivo* use to provide a continuous supply of siRNA. For example, the siRNA can be incorporated in a plasmid or in a viral vector RNA viruses such as lentiviruses provide a means of infecting cells as well as a cloning vehicle.

(d)   providing dsRNA reagents for applications which include: silencing single genes or families of genes in a eukaryotic cell or organism to study function using standard transfection or transformation techniques for nucleic acids;

(e)   reduction of off-target effects compared with synthetic dsRNA preparations by, for example, selecting large dsRNA constructs that are designed to be uniquely representative of the target gene with as little homology as possible to other areas of the genome;

(f)   using these dsRNA fragments as therapeutic agents or in therapeutic agent screening or target validation assays; and

(g)   cloning DNA encoding dsRNA fragments to provide a continuous or *in vivo* regulated supply of gene silencing dsRNA

without the need for *de novo* synthesis for each experiment. Alternatively, the siRNA can be incorporated into the genome of an RNA virus, for example, the lentivirus, for delivery into the host cell.

Other advantages of RNase III mutants such as E38A described herein further include: (i) the ability to obtain the desirable size range of dsRNA products generated by substantially complete digestion of larger dsRNA molecules corresponding to a large portion or the total sequence of the target mRNA so as to circumvent the need for selecting an effective target short sequence; (ii) incubation in standard buffers to facilitate making dsRNA and cleaving it to the desired size all in a single reaction vessel; (iii) enhanced yield relative to wild type RNase III of fragments in the desired size range; (iv) enhanced stability of the fragments obtained using mutant enzymes compared with wild type RNase III in a standard Magnesium buffer; (v) flexibility in time of incubation where a complete reaction can be achieved in as little as 10 minutes although longer incubation times at least up to 16 hours are not detrimental to obtaining the desired reaction products; (vi) reduced cost of gene silencing reagents relative to synthetic products; (vii) opportunities for multiplexing reactions and for generating libraries of fragments; (vii) the ability to form multiple randomly cleaved fragments of dsRNA of about 23bp from fragments having a size as small as about 50 bps; and (viii) the ability to generate fragments of about 23bp from large dsRNA *in vivo* (cell culture or whole organism) by introducing

mutant RNase III by transfection, infection or injection using standard protocols.

The fragmentation of large dsRNA molecules (greater than about 40 bp to at least 10kb) including linear dsRNA or hairpins, provides a population of short RNAs which include multiple effective short sequences (18-30 bp) corresponding to the mRNA for silencing.

Additionally, the advantages of the method described herein obviate the need for calibration of the time of digestion or the amount of enzyme used (Figures 2, 3, 4 and 6) beyond a threshhold value where the threshhold of time is a few minutes and the threshhold of concentration may include a range of 1:1 (w/w) of enzyme:substrate. An optional additional step of removing undesired digestion products by gel electrophoresis or other tedious separation methods can be omitted making the method amenable to automation and suitable for high throughput formats. The RNA starting material can be readily obtained by in vitro enzymatic transcription (NEB catalog, New England Biolabs, Inc., Beverly, MA) or chemical synthesis and can be a double-stranded molecule or a single stranded RNA that forms a double stranded hairpin.

A fraction of the starting material (large dsRNA), for example, at least 30%, more particularly, greater than 40% or 50%, has a size after digestion with mutant RNase III, in the range of 18-30 bp, more particularly, 21-23 bp suitable for gene silencing in cultured mammalian and insect cells. It is expected

that these fragments will also be active in gene silencing in whole organisms such as, plants, microorganisms and animals including humans as well as to cultured cells from the same. Unlike with Dicer, the fragments having a size outside the range of 21-23 nt do not interfere with gene silencing.

In a preferred embodiment, a E38A mutant of RNase III is capable of converting as much as 60% of the substrate to the preferred size range within 10 minutes at 37°C in standard buffer (NEB buffer 2, New England Biolabs, Inc., Beverly, MA). The yield of RNA fragments from digestion of large dsRNA with RNase III E38A mutant may result for example in as much as 2X the yield obtained with RNase III (wt) in the presence of manganese ions and about 10X the yield obtained with RNase III (wt) in the presence of magnesium ions (see, for example, Figure 7).

One of the problems in the field of gene silencing is that of identifying a short dsRNA (15-30 bp) that can achieve the desired goal of effectively targeting a particular messenger RNA, other RNA or gene. In embodiments of the invention, this problem is solved by utilizing a large dsRNA having a sequence that is identical to all or part of the target and cleaving this large RNA into multiple overlapping fragments of the appropriate size for gene silencing. The selection of a suitable sequence for a dsRNA prior to cleavage can be determined for example by the algorithm in Example VI. It is here asserted that the cleavage products are representative of the entire length of the large dsRNA and that the dsRNA fragment preparation is capable of

gene silencing in a variety of cells including insect cells and mammalian cells. Importantly, off-target effects are minimized by the use of hsiRNA mixtures.

5   Once a dsRNA fragment mixture is obtained, it is possible to make a library of clones containing DNA sequences corresponding to individual dsRNA fragments in the mixture. When provided with appropriate promoters, individual clones or mixtures thereof can be used to transfect cells so as to provide a
10   continuous supply of the short dsRNA for use in long-term gene silencing. Silencing of gene expression as a result of transfection of an individual clone or selected mixtures of clones into a target cell or organism may have particular advantages in for example, therapeutic applications, over transient gene silencing effects
15   achieved by transfecting cells with the dsRNA itself. This provides new reagents for therapeutic applications providing an unlimited supply of an agent that specifically modulates gene expression of a particular gene.

20   **Specificity of gene silencing**

          Specificity of gene silencing for a particular targeted mRNA can be confirmed using a BLAST analysis of sequences in the targeted mRNA to determine that no extended regions in the
25   RNA (over 11 bases long) are identical to other gene transcripts to avoid non-specific gene silencing.

          Using the methods described herein, RNA preparations that are specific for a single member of a gene family and do not

silence mRNA from other members of that gene family can be prepared from long dsRNA that is complementary in sequence to a segment of the target mRNA (also referred to as long dsRNA segments). Alternatively, RNA preparations can be prepared that have specificity for any gene in a gene family but do not have specificity for other genes outside the gene family.

The appropriate gene silencing effect may be achieved by targeting mRNA sequences that are unique or that form part or all of a consensus region for a family of mRNAs.

An assay which may be readily used to determine whether a mutant RNase III has improved cleavage activity compared to other enzyme preparations includes an *in vivo* and an *in vitro* test as follows:

(1) *In vivo assay*: dsRNA having a sequence complementary to a transcript of a marker protein is subjected to RNase III digestion to produce a heterogeneous dsRNA fragment preparation. Host cells normally expressing the marker protein are transfected with the RNA fragment preparation and changes in the expression of the marker phenotype determined to ascertain whether a knock down effect in gene expression has occurred as a result of the transfection with the RNA fragment mixture. The *in vivo* assay has been described in more detail in US published application US-2004-0038278 incorporated herein by reference.

(2) *In vitro* assay: dsRNA is subjected to RNase III digestion and the digest run on acrylamide gels to determine the size of fragments produced at various times of incubation and in selected buffers.

The methods described herein can also be applied to producing multiple dsRNA fragment mixtures which can then be used to simultaneously silence multiple genes. Additional uses include targeting upstream or downstream regulatory regions with dsRNA to modulate expression. Accordingly, a heterogeneous population of large dsRNA fragments obtained from transcription of a single DNA template or a plurality of different DNA templates can be digested by mutant RNase III in the presence of magnesium ions.

The above described generation of dsRNA fragment mixtures or clones thereof for making selected siRNA fragments can be achieved in part or as a whole by utilizing a kit of the type described in Example V. Instructions can be provided that include any or all of descriptions of how to make a desired large dsRNA, to generate dsRNA mixtures and to transfect cells with such mixtures.

Illustrative Uses

The availability of hsiRNA fragments provides a supply of a reagent or therapeutic agent and a novel therapeutic approach in which a desired knockdown effect can be achieved in a whole organism without the disadvantages of gene therapy. However,

clones expressing siRNA fragments or dsRNA mixtures can be used for complete, modulated or transient *in vivo* silencing of a target gene.

A gene derived from a pathogen can be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen or maintenance of the infection. The inhibitory RNA could be introduced in cells *in vitro* or *ex vivo* and then subsequently placed into an organism to effect therapy, or the organism could be directly treated by *in vivo* administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

Embodiments of the present invention may be applied to treatment or development of treatments for cancers of any type, including solid tumors and leukemias.

Embodiments of the present invention are exemplified as follows. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

5

All references cited herein are incorporated by reference, including U.S. provisional applications 60/538,805, 60/572,240 and 60/543,880.

10   **EXAMPLES**

Example I: Preparation of *E. coli* RNase III mutants

All *E. coli* RNAse III mutants (except for E117D which was
15   generated by mutagenesis using PCR were constructed by a
standard 2 step PCR sewing technique (*Methods Enzymol*. 185:
60-89 (1990)). Other cloning techniques used herein are
standard in the art and can be found in Sambrook, J., Fritsch, E.
F., Maniatis, T. "Molecular Cloning: A Laboratory Manual", Cold
20   Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
The starting plasmid was *E. coli* RNAse III cloned into pET16B
(EMD Bioscience, Inc., San Diego, CA), which produced a His-
tagged RNAse III protein under control of a T7 promoter.

25   <u>WT RNAse III</u>

E. coli RNAse III was amplified from a pMalE/RNAse III clone
with the following primers:

ACAGGATCCCATGAACCCCATCGTAATTAAT (SEQ ID NO:1)
ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT (SEQ ID NO:2)

The PCR product was cleaved with BamHI and cloned into
the BamHI site of pET16b, resulting in a plasmid that
synthesizes His-tagged WT RNAse III. This His-tagged WT
RNAse III clone in pET16b was used as a substrate in PCR
reactions to create all amino acid substitution mutations (except
for E117D) as described below.

Formation of E38A mutant

The primers used to construct the carboxy terminal half of
E.coli RNase III (Accession No. X02946) with an E38A mutation
were:

CAGTAAACATAACGCGCGTTTAGAAT (SEQ ID NO:3)
ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT (SEQ ID NO:2)

The primers used to construct the amino terminal half of
RNAse III with an E38A mutation were:

AATTCTAAACGCGCGTTATGTTTACT (SEQ ID NO:4)
TAATACGACTCACTATAGGG (SEQ ID NO:7) (NEB primer cat#
1248 (New England Biolabs, Inc., Beverly, MA).

The two PCR products were then 'sewn' together in one
PCR reaction using both as substrates and primers (SEQ ID
NOS:7 and 2) for the reaction. The resulting product was then

cleaved with Xba I and cloned back into a modified pET16b at an XbaI site, resulting in a His-tagged RNAse III with an E38A mutation.

5   Formation of E65A RNase III

E65A RNAse III was constructed in a similar two-step process. In the first step, the His-tagged RNAse III plasmid was amplified with the following primer sets in two PCR's:

10

CTCGTGTGGATGCAGGCGATATGAGCCGGAT (SEQ ID NO:5)
ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT (SEQ ID NO:2)
to amply the 3' end of the gene.

15   TCCGGCTCATATCGCCTGCATCCACACGAGGGA (SEQ ID NO:6)
TAATACGACTCACTATAGGG (SEQ ID NO:7) (NEB primer cat#
1248 (New England Biolabs, Inc., Beverly, MA) to amplify the 5'
end of the gene.

20   The two PCR products were then used as substrates in a subsequent PCR reaction and cloned into the XbaI site of the modified pET16b vector described in I.B.

Formation of additional RNAse III mutants

25

Any specific point mutation can be constructed from the cloned wt RNAse III gene by a similar method to the one described to create both the E38A and E65A mutants. In the first step, two primers, one for the coding strand and one for the

non-coding strands, are designed such that they introduce the desired mutation at the selected amino acid. The primers are of sufficient length and span such that they would still anneal to the substrate given the mismatch necessary to introduce the mutation. The primer for the coding strand is matched with an appropriate downstream primer on the opposite strand in PCR that amplifies this region of the RNAse III gene. A similar PCR is performed using the non-coding primer and an appropriate primer upstream on the opposite strand that results in an overlapping upstream fragment of the RNAse III gene. The two fragments are 'sewn' together using the two fragments as substrates and the outside primers. The resulting RNAse III gene now contains the point mutation desired and can be cloned back into the appropriate vector. All mutations with the exception of E117D were constructed in this way.

The E38 residue of the *E. coli* RNAse III gene corresponds to the E37 residue of the *Aquifex aeolicus* RNAse III gene. Likewise D45 corresponds to D44, E65 corresponds to E64, E117 to E110. This was determined by several standard multiple sequence alignment software packages. The corresponding amino acid residues can be determined for any RNAse III gene by similar methods.

Example II: Production and purification of RNase III mutants

Expression & Purification

5

30 ml cultures of each mutant and WT clones were grown in *E. coli* ER2566 (New England Biolabs, Inc., Beverly, MA) to mid log phase, then induced by the addition of IPTG to a final concentration of 100 μM and shaken at 15°C overnight. Induced

10   cultures were lysed by sonication.

The RNase III mutants were purified from the cleared lysates by Qiagen Nickel resin affinity purification (according to manufacturer's instructions) and assayed by standard methods.

15   The enzyme reaction was performed in NEB Buffer 2 (New England Biolabs, Inc., Beverly, MA), at 37°C, for 1 hr using 0.5 μg of a 900 bp dsRNA as a substrate. The product of the reaction was analyzed by non-denaturing polyacrylamide electrophoresis.

20   RNase Activity Assay

0.5 μg of MalE dsRNA (HiScribe kit-NEB, New England Biolabs, Inc., Beverly, MA) was digested with RNAse III (wt and mutants in the range consistent with optimal conditions in a 20

25   μl reaction mixture at 37°C in NEB Buffer 2 (0.05 M NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM MgCl$_2$, 1 mM dithiothreitol) (New England Biolabs, Inc., Beverly, MA) for 1 hour.

-36-

As can be seen in the Figure 3A, the end product of digestion of dsRNA with E38A is predominantly a dsRNA fragment of about 23 bps in length (lanes 7, 8, and 9). This product appears in as little as 1 minute and the reaction is 'complete' by 10 minutes (Figure 6). In addition the 23 bp product is stable for at least 16 hrs (Figure 3B, lanes 7-9) and is slowly lost over several days (Figure 5).

The activity of E65A (Figure 10) is in direct contrast to what was reported in (Blaszczyk, J., et al. *Structure* 9:1225-1236 (2001)). In this paper the authors describe an E65A mutation of RNase III as disabling RNAse III function. By our assay, E65A produced a similar product to E38A (Figure 10, lanes 6-8). However, the product appears to be less stable and has disappeared by 16 hrs (Figure 10B, Lanes 4 & 6).

Example III: dsRNA cleavage and Gene Silencing Activity in cultured cells.

To test the ability of the dsRNA product of RNase III mutant digestion to induce RNA interference, an *in vivo* assay according to US published application US-2004-0038278 was carried as follows: dsRNA made from firefly luciferase encoding DNA sequence was cleaved with the wt RNAseIII (Shortcut) or E38A mutant RNase IIIs (Figure 9). NIH 3T3 cells were transfected with a reporter plasmid expressing the firefly luciferase gene, another reporter acting as a transfection control and dsRNA. Cells transfected with the reporters and no dsRNA or unrelated digested dsRNA show significant luciferase activity.

The cells transfected with hsiRNA from wild type or mutant Rnase III show a knockdown of luciferase activity. The results are shown in Figure 9.

Example IV: The cleavage products of E38A RNase III on dsRNA is a set of heterogeneous fragments that are overlapping.

Short dsRNA cleavage products of mutant RNase III digestion contain sequences representing the entire parent sequence. This is determined by cloning and sequencing the dsRNA cleavage products or by hybridization to the template DNA sequence fragments. Generation of a library of cloned RNase III products is readily achieved. The techniques used here to demonstrate the presence of overlapping fragments are the same as those described in US published application US-2004-0038278 herein incorporated by reference.

Example V: Kits for generating hsiRNA and for gene silencing in mammalian cells

A kit is provided for *in vitro* generation of dsRNA mixtures and optionally for transfection of RNA fragments into mammalian cells.

In an embodiment of the invention, each kit contains reagents for processing multiple large dsRNAs for transfection of cells and includes instructions for use.

<u>Kit Components</u>

        The kit may contain enzyme and optionally vectors,
5   primers and/or buffers. The kit may include one or more of the
    reagents listed below (each obtainable from New England
    Biolabs (Beverly, MA)).

        T7 RNA Polymerase, 150 units/µl,   25 µl
10 10X Buffer/NTPs (see formulation below)   60 µl
    30X High Molecular Weight Component Mix
                (HMW) (see formulation below)   20 µl
    BT7-minimal Primer (19 MER),
            5′-Biotin-dCTCGAGTAATACGACTCACTATAGG-3′,
15   (SEQ ID NO:26) (10 µM)   25 µL
    10X Mutant Ribonuclease III (1.4 µg/µl)   100 µL
    10X hsiRNA Buffer (see formulation below)
    10X EDTA (250 mM)   1000 µL
    Litmus 38iluc control template,   1 µg
20 RNase-free glycogen 10 µg/µL   50 µL
            Plasmid DNA 500 µg/ml in TE buffer (10 mM Tris-HCl, pH
            8.0, 1 mM EDTA)

        In addition, the kit may include transfection reagents, RNA
25 size markers and Streptavidin-coated beads.

<u>Buffer compositions</u>

    (a)   10X Buffer/NTPs:
            400 mM Tris-HCl, pH 8.1
30   190 mM $MgCl_2$
            50 mM DTT
            10 mM spermidine
            40 mM each NTP

35 (b)   30X High Molecular Weight (HMW) Mix:
            20 mM Tris-HCl, pH 8.1

1.5 mg/ml BSA
100 units/ml inorganic pyrophosphatase (yeast)
12,000 units/ml pancreatic ribonuclease inhibitor
50% glycerol

(c)   10x hsiRNA buffer
0.5M Tris-HCl, pH 7.5
0.5M NaCl
0.1M MgCl₂
10mM DTT

The kit utilizes mutant RNase III in an optimized buffer to produce fragments in the range of about 18-30 nt (more particularly 21-23 nt) from long dsRNA. The dsRNA product is cleaved with mutant RNase III to reproducibly yield dsRNA mixtures suitable for silencing gene expression. The sequences of different siRNA fragments in the mixture map to sequences along the entire target gene. The dsRNA mixtures can be purified by ethanol precipitation and used in transfection.

An example of instructions accompanying the kit include the following:

(1)   Cloning the DNA template prior to *in vitro* transcription to generate dsRNA

One approach to making a DNA template for transcription is to clone a DNA of interest in Litmus 28i/38i bi-directional transcription vectors (New England Biolabs, Inc., Beverly, MA). The DNA of interest can then be amplified by PCR using a single T7 promoter-specific primer such as a BT7 Minimal Primer which

produces a linear product with the target sequence flanked by T7 promoters which define the ends.

Alternatively target gene-specific primers with appended T7 promoters can be used to amplify any specific cDNA sequences. For example, the amplification primer:

5'<u>TAATACGACTCACTATAG</u>**aaggacagatggttaagtac**-3'
        T7 promoter   (SEQ ID NO:8)

in which a T7 promoter (underlined) located at the 5' end preceding the target-specific sequence (bold) can be used for amplifying any cDNA template.

Biotinylated BT7 primer can be used to amplify any sequence flanked by T7 promoters. Optionally, the amplified biotinylated DNA template can be isolated by binding to streptavidin magnetic beads (New England Biolabs, Inc., Beverly, MA) and used directly as a template for transcription. For forming an immobilized DNA template, 25-50 μL of streptavidin magnetic bead suspension is added to the amplification (PCR) reaction mix with an equal volume of 1 M NaCl and incubate at room temperature for 10-15 minutes. The supernatant is removed in the presence of a magnet and the beads washed with 0.5 mLs TE, 0.5 M NaCl. The resuspended beads can be used directly in the transcription reaction. *In vitro* transcription of the immobilized DNA template produces DNA-free dsRNA.

Amplification can be achieved by any polymerase dependent method such as PCR. The amplification product is purified by ethanol precipitation, or by a chromatographic method (e.g., QiaQuick® column (Qiagen, Studio City, CA)) and

5   resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, prepared with Milli-Q water or equivalent) to a final concentration of ~500 µg/ml.

A control consisting of GL3 luciferase can be prepared

10   using a Litmus 38iLuc plasmid in which a 1.0-kbp fragment of the GL3 luciferase gene is cloned in the SphI and NgoMIV sites of Litmus 38i. Linearization with MfeI and StuI (in separate reactions), followed by *in vitro* transcription of the combined linearized templates, produces dsRNA 1.0 kbp in length.

15

Pilot studies can be undertaken for providing an hsiRNA mixture for specific gene silencing using one or more fragments obtained by cleaving dsRNA having a length of 100-600 bp including RNA derived from restriction fragments of a cDNA

20   which has been subcloned into Litmus28i/38i vectors (New England Biolabs, Inc., Beverly, MA).

In vitro Transcription

25   *In vitro* transcription is performed using the DNA template prepared as described above. The volume of template used in the transcription reaction depends on the method of purification. For unpurified PCR product, no more than 5 µl is used per 30 µl

reaction. The amount of added template DNA should not exceed 1 µg per 30 µl reaction.

5

RNase-Free Water	50 - x µl
10X Buffer/NTPs	6 µl
DNA template (~0.5-1 µg)	x µl
30X HMW Mix	2 µl
T7 RNA Polymerase (150 U/µl)	2 µl
	60 µl

10

Incubation at 42°C can improve yields of RNA transcripts containing substantial secondary structure. As it is difficult to gauge the secondary structure content in a particular transcript,

15   we recommend that all transcription be carried out at 42°C if possible. Transcription yields increase linearly for the first 90 minutes (approximately) and reach maximum after 2-3 hours. Reactions can be carried out overnight if desired, but yields will not be higher. Double-stranded RNA is stable upon prolonged

20   incubation at 37°C.

The transcription reaction can be analyzed on a 1% agarose gel taking care to avoid RNase contamination. Double-stranded RNA migrates approximately as the DNA template used

25   in the reaction. The expected length of the transcript from the Litmus 38iluc control template is 1000 bp.

The dsRNA transcription product is purified by ethanol precipitation. One-tenth volume of 3 M NaOAc is added at a pH

30   5.5 with 2 volumes of cold 95% ethanol. Incubate on ice for 15 minutes, or store at –20°C overnight. Spin for 15 minutes in a microcentrifuge at 14,000 rpm. Remove supernatant, add two

volumes 80% ethanol, incubate at room temperature for 10 minutes, centrifuge for 5 minutes, and decant and drain the tube. Allow the pellet to air-dry. Dissolve the dried RNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, or dH_2O.

5

Forming an dsRNA mixture

Use 1X (10-fold diluted) RNase III at a concentration of (0.14 µg/ul) and 0.07 µg/µL of dsRNA in the digestion reaction as in the following example.

10

Combine the following:

dH_2O	120 - x µL
10X hsiRNA Buffer	15 µl
dsRNA	x µL (10 µg)
mutant RNaseIII	15 µl
	150 µl

20

Incubate for 10 min at 37°C.
Promptly add 15 µl 10X EDTA to stop the reaction.

For monitoring the products of digestion, a 10-20% native polyacrylamide gel is suitable. The product of digestion reveals that the long dsRNA has been cleaved to yield an dsRNA mixture of fragments having a size in the range of 18-25 nucleotides regardless of the length of the starting long dsRNA. The mixture can be purified by the single step of ethanol precipitation prior to use in transfection.

25

30

Ethanol precipitation of hsiRNA fragments

Add one-tenth volume of 3 M NaOAc, pH 5.5, 2 μL glycogen solution and 3 volumes of cold 95% ethanol. Place at −70°C for 30 minutes, or −20°C for 2 hrs-overnight. Spin for 15 minutes in a microcentrifuge at 14,000 rpm. Remove supernatant carefully avoiding the small pellet, add two volumes 80% ethanol, incubate at room temperature for 10 minutes, centrifuge for 5 minutes, and decant and drain the tube. Allow the pellet to air-dry. Dissolve the dried RNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, or dH_2O.

Determining dsRNA concentration

This can be measured using a UV spectrophotometer (OD at 260 nm of 1 corresponds to 40 μg/mL dsRNA) or a fluorometer (using RIBOGREEN®, Molecular Probes, Eugene, OR) or comparisons to siRNA standards used in the art.

Transfection guidelines

After ethanol precipitation, dsRNA mixtures can be directly transfected into mammalian cells using reagents and protocols suitable for oligonucleotide transfections such as lipofectin 2000, oligofectamine, TRANS-IT TKO® (Mirus Corp., Madison, WI) and Targefect (Targeting Systems, Santee, CA) or trnasfection reagents provided by New England Biolabs Inc. (Beverly. MA). Additionally, Calcium Phosphate and Electroporation have been reported to be efficient in transfecting short RNAs.

-45-

Amounts of 25-100 ng of dsRNA can be used per transfection well (24-well format) as an initial amount to be adjusted according to experimental findings.

5   Large dsRNA may be synthesized by *in vitro* transcription as described above using a modified transcription buffer containing modified ribonucleotides in place of NTPS in 10X bufffer described above such as 2-fluoro-ribo-CTP, 2-fluoro-ribo-UTP, 2-O-methyl-ribo-CTP, 2-O-methyl-ribo-UTP, 2-O-methyl-

10   ribo-ATP, 2-O-methyl-ribo-GTP or other 2' modifications that render the dsRNA more stable or resistant to degradation. A DURASCRIBE® kit (Epicentre Technologies, Madison, WI) may be used for these purposes.

15   **Example VI: Specific siRNA mixtures**

Segments of mRNA sequence for an identified target can be selected subsequent to a sequence comparison with the gene database conducted with each target sequence.

20

An algorithm is used to scan the sequence using a window of, for example, 16-21 bp. BLAST scores can then be obtained for each segment after alignment with a complete database (such as UNIGENE). Those entries calculated from the BLAST scores

25   that are higher than a set limit were eliminated from the report. Regions of the target sequence showing absence of hits to other targets are preferably selected.

Although there is no particular limitation on segment size, segments may be selected in the range of about 150-1000 bp for example, 200-400 bp long.

5   A selected DNA segment corresponding to target dsRNA can be amplified using PCR primers that amplify efficiently using standard protocols for PCR primer design. For example, primers may include a T7 promoter sequence at the 5′ end. Other sequences can be used in place of the T7 promoter to facilitate
10   cloning to one of the double T7 promoter vectors (Litmus 28i, Litmus 38i, Litmus-U from New England Biolabs, Inc., Beverly, MA).

For Litmus U, the following primer sequences can be used:
15   gggaaagu and ggagacau, where u stands for uracil. After the PCR reaction, the amplified DNA product can be cloned directly in Litmus U using the USER protocol (New England Biolabs, Inc., Beverly, MA). The cloned fragments may then be used for the production of dsRNA (see for example US published application
20   US-2004-0038278).

Each sequence determined to be suitable for use in preparing hsiRNA is described by accession number and sequence in Table 1.

25
Preparation of hsiRNA mixtures is described in Example II.

The functionality of the hsiRNA mixtures can be determined by transfecting cells as described in Example III. In

addition, Western blot or protein activity assays can be
performed at time points after transfection to determine
whether gene expression has been down regulated.

5   Table 1

CATEGORY	TARGET	Acc. number	Coordinates
kinases	Akt1	NM_005163	199-657
	Erk2	NM_002745	660-940
	MSK1	AF074393	282-736
	p38	L35253	10-419
	IRS1	NM005544	1026-1713
	PKR	M35663	999-1499
	PTEN	NM_000314	1019-1445
transcription	CREB	M34356	247-601
Nuc. signaling	ERa	NM_000125	369-905
	ERb	NM_001437	587-1240
	DAX	NM_000475	1-249
	p53	NM_000546	717-915
	DNMT1	X69632-G-BPR2	2124-3235
	DnMT3B	AF331857	1150-1545
	DnMT3A	X63692.gb-pr2	1547-2388
	TRIP	L38810	1-445
	Rb	m15400.gb_pr1	2239-2755
	MeCP2	af030876.gb_pr	699-1011
Other	caspase3	p42574	1063-1496
	La	NM_003142	316-631
	FURIN	NM002569	1781-1990
Controls,	Lit28i polylinker	NEB#N3528S	2465-2600
gen. use	EGFP	U55763	596-1322
	RFP	AF272711	152-632
	FfLUC	U47295	747-1757
	Renilla	AF264722	3673-3951

Table 1 lists examples of target genes for which hsiRNA
fragments can be prepared using mutant RNaseIII as described
10   herein and used for gene silencing in cells that are cultured or

occur *in vivo*. The coordinates for the gene (cDNA) sequence are contained in accession number of GenBank given above.

Algorithm for selection of suitable RNA sequences for forming an hsiRNA mixture.

1. The target sequence SO of length N is input from the user.

2. All possible oligonucleotide subsequences (S_1, S_2, S_3..$S_{(n-L+1)}$) of a user-specified length L are extracted from SO.

3. The nucleotide composition of each oligonucleotide subsequence is calculated.

4. The thermal stability or free energy of helix formation is calculated along each oligonucleotide subsequence. For purposes of this calculation, the sequence is treated as a double stranded RNA duplex with 2 base 3' overhangs.

5. (optional) The differential stability of the 2 ends of the double stranded RNA duplex is calculated. Differentials where the stability of the 5' sense strand end are marked as favorable. The opposite differential is marked unfavorable.

6. A database of sequences representing the transcriptome of the user-selected target organism is queried with sequence SO to identify sequences with a user-specified level of similarity to SO. These similar sequences are marked as equivalent to the target sequence and their identities are recorded.

7. The same transcriptome database of the user-selected target organism is queried to determine the frequency of occurrence of each oligonucleotide subsequence within the transcriptome. An occurrence of the oligonucleotide subsequence is recorded if the oligonucleotide subsequence has a user-specified level of

similarity to a subsequence from the transcriptome database. For each occurrence, the identity of the matching segments within the transcriptome is recorded as well. These matching segments are termed "cross target" or "off target" matches.

Sequences identified as equivalent to the target SO in the preceding step are filtered out and not counted as cross target matches.

8. For each oligonucleotide subsequence, data representing the frequency of of cross-target matches in the transcriptome , the nucleotide composition and the differential thermal stability of its ends are presented.

9. Generally, regions with low cross-target frequencies, moderate %GC composition and high density of favorable differential thermal stability are considered desirable regions for subsequent primer selection.

In the current embodiment of this algorithm, the megablast program from NCBI which uses a variant of the BLAST algorithm is used to search the transcriptome database. The similarity measure used to identify cross-targets is the presence of a user-specified minimum number of contiguous nucleotide matches between the target oligonucleotide subsequence and the subject sequence in the transcriptome database. The nucleotide composition measure displayed is %GC. Thermal stability at the ends of the oligonucleotide subsequence is calculated using the 4 terminal base paired nucleotides and excludes the unpaired overhanging sequence.